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Influence of yeast quality on performance of gnotobiotically grown *Artemia*

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Abstract

Using axenically grown *Artemia*, a model system was developed to evaluate the effect of bacteria on the survival and development of this crustacean. Two strains of baker's yeast (*Saccharomyces cerevisiae*) were used in all experiments as feed for *Artemia*: a wild-type strain and its mnn9 mutant, defective in the synthesis of mannoproteins in the outer cell wall. The genetic background, yeast growth phase and growth medium appeared to be important parameters determining the quality of yeast cells as feed for *Artemia*. A strong positive correlation between *Artemia* performance and the yeast cell wall chitin and glucan content was obtained, while the mannoprotein content was negatively correlated. Mnn9 yeast cells grown till exponential phase in minimal medium proved to be excellent feed for *Artemia*, yielding an average 95% survival and 4-mm growth after 6 days at 28 °C, which is comparable to the best results obtained with algal feed. The standard growth test yields highly reproducible results and can become an excellent tool to study the mode of action of bacteria. Furthermore, yeast cell viability and the method used to kill/sterilize the cells are important parameters influencing nauplii performance.
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1. Introduction

Intensive culture of *Artemia* as a live prey for fish and shrimp larvae has always suffered from unpredictable results due to incidental crashes in individual production

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tanks. The associated microflora plays an important role in the instability and variability of live feed cultures (Verdonck et al., 1994; Verschuere et al., 1997), as well as in cultures of their aquatic predator larvae (Perez-Benavente and Gatesoupe, 1988). Intensive rearing of live prey often requires that feed is provided at a high concentration, resulting in an excellent medium for the growth of heterotrophic and/or opportunistic pathogenic bacteria.

Until recently, only antibiotics were considered for controlling high mortalities in aquaculture. This practice is now questioned and often banned because of environmental and human health consequences (Witte, 2000). Several authors have proven that new zootechnical methods, such as the use of selected microbiota (e.g. "mature water", specific microalgae, probiotic yeasts and bacteria), may constitute alternative approaches for successful and reproducible larviculture (see review of Verschuere et al., 2000a).

Although the importance of a proper control of the bacterial community in live feed and fish larvae production is now generally acknowledged, the exact role and mode of action of probiotic bacteria is less well studied (Verschuere et al., 2000a).

A major handicap in studying the role of bacteria and probiotics on aquatic organisms is the difficulty to eliminate (or distinguish the effect of) the microflora that is normally present in any aquatic environment and hence to determine its exact role.

Artemia is an excellent model organism to study the mode of action of probiotic bacteria, as it can easily be cultured in axenic conditions and used as a vector for the transfer of probiotics to larvae of target-species. In order to study the role of bacteria in the production of *Artemia*, a standardized culture system yielding predictable and reproducible results is necessary.

A wide variety of feeds can be used to culture *Artemia* due to the particular feeding characteristics of this animal: *Artemia* are continuous, nonselective, particle-filtration feeders. Successfully used feed sources include live microalgae, such as the genus *Dunaliella* (Mason, 1963), *Isochrysis* (Wickins, 1972), *Tetraselmis* (Fábregas et al., 1996) and *Chaetoceros* (Juarez Carrillo, 1995; Naegel, 1999), as well as dried microalgae, such as the genus *Spirulina* and *Scenedesmus* (Person Le Ruyet, 1976). The production of unicellular algae, however, is labor-intensive and expensive. Therefore, several substitutes have been used, such as bacteria (Intriago and Jones, 1993), baker's yeast (Coutteau et al., 1990), soybean, rice, wheat bran (Dobbeleir et al., 1980), nestum (Naegel, 1999), cerophyl and lactoserum (Douillet, 1987). These substitutes are interesting alternative feeds for *Artemia* since they have a small particle size, a high protein content, good buoyancy in the water column and low production costs (Coutteau et al., 1990).

A common trait of all these feeds is the fact that they are utilised under plain xenic conditions. This implies that in most cases there is no way to distinguish between the nutritional contribution of the often prolific but fortuitous positive critical role of bacteria for certain *Artemia* feeds (e.g. ricebran), from the feed itself (Douillet, 1984). Only under axenic conditions can the real value of a feed be appreciated.

The present study investigates the use of the baker's yeast *Saccharomyces cerevisiae* as feed for axenically grown *Artemia*. Several parameters were studied, namely the yeast growth phase, the yeast culture medium, the genetic background and the viability. The results indicate that all these parameters have a strong influence on the culture performance of *Artemia*. By controlling these parameters *Artemia* can be grown axenically with strongly diverging, but highly reproducible results for each set of parameters.

2. Materials and methods

2.1. Axenic yeast culture

Two different strains of baker's yeast *S. cerevisiae* were used as feed for *Artemia*: the wild-type strain (WT) (BY4741, genotype *Mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and its mnn9 isogenic mutant (BY4741; genotype *Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*; YPL050c::kanMX4), which has a null mutation resulting mainly in a lower concentration of mannoproteins in the outer layer of the cell wall (the mnn9 mutation prohibits mannose chain extension) (Magnelli et al., 2002). Both strains were provided by EUROSCARF, University of Frankfurt, Germany.

Yeast cultures were grown in sterile Erlenmeyers with a cotton cap placed on a shaker in the dark (30 °C; 150 rpm). Both strains were cultured in two different media: a complete Yeast Extract Peptone Dextrose medium (YEPD) (the most common and complete medium used to culture yeast) containing yeast extract (Sigma, 1% w/v), peptone bacteriological grade (Sigma, 1% w/v) and D-glucose (Sigma, 2% w/v); and a minimal Yeast Nitrogen Based medium (YNB) with amino acids (histidine HCl: 10 mg/l; methionine: 20 mg/l; tryptophan: 20 mg/l; inositol: 2 mg/l—Sigma, 0.67% w/v), supplemented with uracil (Sigma, 0.002% w/v), L-leucine (Sigma, 0.002% w/v) and D-glucose (Sigma, 0.5% w/v). These media were prepared in natural seawater (35 g/l) that was previously filtered (0.22 μm) and sterilized by autoclavation at 120 °C for 20 min.

The growth curve of each yeast strain, cultured in a specific medium, was established by regularly measuring their absorbance at 600 nm with a spectrophotometer. Both strains were harvested by centrifugation (3000 rpm for 10 min), either in the exponential growth phase (after 20 h; further on called "exp-yeast") or in the stationary growth phase (after 3 days; called "stat-yeast"). Cells were resuspended twice in sterile Falcon tubes (TRP®, γ-irradiated) with 20 ml of filtered (0.22 μm) and autoclaved seawater (FASW). All handlings were performed in a laminar flow hood to maintain sterility.

Yeast density was determined by measuring twice the cell concentration, using a Bürker haemocytometer. Yeast suspensions were stored at 4 °C and used to feed *Artemia* until the end of each experiment.

For experiments 2 to 5, the YEPD-grown WT and mnn9 strains that were offered dead to *Artemia* were killed with 10-kgy gamma-irradiation (γ-irradiation) provided by a 15-MeV 20-kW linear electron accelerator or with 20% norvanol D (Merck Eurolab; composed by 90% v/v ethanol, 2.9% v/v ether; 9% m/v H_2O) (incubated for 30 min at 28 °C, centrifuged twice to remove norvanol residues and replaced with FASW) or with high temperature (autoclavation at 120 °C for 20 min). In order to check if all yeast cells were effectively killed by the three methods, both yeast strains (cultured on YEPD and harvested in the exponential and stationary growth phase) were plated after being exposed to each method, by transferring 100 μl of culture medium to YEPD agar plates (three replicates). The YEPD agar has the same composition of YEPD medium with the addition of agar bacteriological grade (Sigma, 2% w/v). Absence of yeast growth was monitored after incubating the plates for 5 days at 28 °C.

2.2. Yeast ash free content, fatty acid composition and cell wall composition

To determine the yeast ash free dry weight (AFDW), 50 ml of each culture sample was filtered on pre-dried nitrocellulose filters (pore size 0.45 μm, two replicates per culture). Filters were subsequently dried at 60 °C for 48 h and weighed. Afterwards, they were combusted at 600 °C for 6 h to determine the ash content. The AFDW was calculated as the difference between the dry weight and the ash weight. The DW and AFDW of the control (filter only, $n=2$) were subtracted from all samples.

To determine the levels of fatty acids contained in the lipid fraction of each yeast culture, approximately 2 g wet weight of cells was harvested for fatty acid methyl ester (FAME) extraction, conducted as described by Coutteau and Sorgeloos (1995).

To analyse the cell wall composition of yeast cultured in different media and harvested in different growth phases, approximately 0.25 g dry weight of each culture was harvested, centrifuged and freeze-dried (with three replicates). The quantification of chitin, mannan, β-glucan concentrations was determined, using high performance anionic exchange chromatography (HPAEC) (Dionex Bio-LC50 system, Sunnyvalle, USA), according to the methodologies described by Dallies et al. (1998).

2.3. Axenic Artemia culture

In previous studies, axenic *Artemia* were obtained either by using antibiotics (Gomez-Gil et al., 1998), merthiolate (Gibor, 1956; Provasoli and Shiraishi, 1959; Sick, 1976; Rico-Mora and Voltolina, 1995; Douillet, 1987; Verschuere et al., 1999) or hypochlorite-mediated decapsulation (Orozco-Medina et al., 2002).

In the present study, experiments were performed with high hatching quality cysts of *Artemia franciscana*, originating from Great Salt Lake, Utah, USA (EG® Type, INVE Aquaculture, Belgium). Bacteria-free cysts and nauplii were obtained via decapsulation, using the procedure described by Sorgeloos et al. (1986). Decapsulation is a process where the chorion of the cysts is removed by immersion in a hypochlorite solution for a few minutes. During decapsulation, 0.22-μm filtered aeration was provided. All manipulations were carried out under a laminar flow hood and all tools were previously autoclaved at 120 °C for 20 min.

Decapsulated cysts were washed carefully with FASW on a 50-μm pore size sterile net and transferred to a sterile 50-ml screw cap Falcon tube containing 30 ml of FASW. The tube was capped and placed on a rotator at four cycles per minute. Cysts were exposed to constant incandescent light (2000 lx) and temperature of 28 °C for 18–20 h.

In the laminar flow, 20 hatched nauplii (Instar II) were transferred to new sterile 50-ml Falcon tubes containing 30 ml of AFSW, together with the amount of feed scheduled for day 1. After feeding, all Falcon tubes were put back on the rotator. Tubes were transferred to the laminar flow just once per day for feeding. The daily feeding schedule was adapted from Coutteau et al. (1990), who optimised the feeding schedule of *Artemia* using baker's yeast as feed. The feeding schedule is intended to provide ad libitum ratios, but avoiding excessive overfeeding in order not to affect the water quality in the test tubes. In each experiment, the amount of yeast cells added

per day and per Falcon tube was: day 1 (9×10^6 cells), days 2 and 3 (1.8×10^7 cells), day 4 (2.4×10^7 cells) and day 5 (3.6×10^7 cells).

2.4. Methods used to verify axenity

Axenity of feed, decapsulated cysts and *Artemia* culture at the end of each experiment were checked by using a combination of plating and live counting. Absence of bacteria was monitored by transferring 100 μl of culture medium to Petri dishes with marine agar 2216 (two replicates). Plates were stored in an incubator for 5 days at 25 °C. As for the live counting, each sample was stained with tetrazolium salt MTT (-3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma, 0.5% w/v) in a sterile recipient (1 part of MTT to 9 parts of sample) and incubated at 30 °C for 30 min. Under a light microscope (1000 × magnification), live bacterial detection and counting were performed. The MTT staining is based on succinate dehydrogenase that is present in viable cells and that metabolises the yellow, water-soluble MTT into a blue, insoluble formazan derivative (Sladowski et al., 1993). The amount of formazan blue-stained cells is proportional to the number of living/viable cells present in a culture (Sladowski et al., 1993). Whenever a culture tube was found to be contaminated, the data were not considered for further analysis and the treatment was repeated if necessary.

2.5. Experimental design

In the first experiment, live WT or mnn9 yeast strains cultured in YEPD (complete medium) or YNB (minimal medium), and harvested in the exponential or stationary growth phase, were used as feed for *Artemia*.

In experiments 2, 3, 4 and 5, live or dead yeast (killed with 10-kgy γ-irradiation, or 20% of norvanol-D or autoclavation) was used as feed for *Artemia*. In experiments 2 and 3, the WT strain harvested in the exponential or stationary growth phase was used. In experiments 4 and 5, exp-grown or stat-grown mnn9 yeast cells were used as feed for *Artemia*.

Each feed was tested in four replicates. All experiments were performed twice (A and B) to verify the reproducibility of the results.

2.6. Survival and growth of Artemia

At the end of each experiment (day 6 after hatching), the number of swimming larvae was determined and the survival percentage was calculated. Living larvae were fixed with Lugol's solution, allowing to measure their individual length (growth calculation), using a dissecting microscope equipped with a drawing mirror, a digital plan measure and the software Artemia 1.0® (Marnix Van Domme). As a criterion that combines effects of survival and growth, the total length (or total biomass production) was determined according to the following equation:

$$\text{Total length (millimeters per Falcon tube or mm/FT)} = \text{number of survivors} \times \text{mean individual length}$$

Values of larval survival (percentage) were ArcSin-transformed, while values of individual length and total length were logarithmic transformed or square root transformed to satisfy normal distribution and homocedasticity requirements. Differences on survival, individual length and total length of *Artemia* fed with different feeds were investigated with analysis of variances (ANOVA) and with multiple comparisons Tukey's range. Correlations between *Artemia* total length and the analysed feed parameters (yeast cell wall percentage, yeast AFDW and percentage of mannan, glucans and chitin present in the yeast cell wall) were also performed. The statistical analysis was done at 0.05 level of probability using the software Statistica 5.5® (Statsoft).

3. Results

3.1. Performance of Artemia fed live yeast cells

3.1.1. Effect of yeast genetic background

Compared to the WT yeast strain, *Artemia* fed with the mnn9 yeast strain, which is defective in mannose chain elongation for outer cell wall-associated mannoprotein, always gave significantly higher total biomass production (total length) (in all eight experiments, irrespective of the growth phase and medium composition) (Tables 1 and 2—Experiment 1). This difference was due to the significantly higher *Artemia* survival as well as average individual length. (in seven out of eight experiments).

3.1.2. Effect of yeast growth stage

Only in five out of eight experiments, higher total biomass production was obtained when exponentially grown yeast cells were fed to *Artemia* compared to nauplii fed

Table 1
Experiment 1—average survival (%), individual length (mm) and total length (mm per Falcon Tube—FT) of *Artemia* nauplii fed live yeast cells: effect of media composition, growth stage and genetic background ($n=4$)

Yeast treatments	A			B		
	Survival (%)	Individual length (mm)	Total length (mm/FT)	Survival (%)	Individual length (mm)	Total length (mm/FT)
WT exp YEPD	15 ± 4^{d}	2.3 ± 0.2^{cd}	6.6 ± 1.2^{c}	18 ± 6^{de}	2.3 ± 0.5^{de}	7.8 ± 2.7^{e}
WT stat YEPD	3 ± 3^{d}	1.7 ± 0.1^{e}	0.9 ± 1.0^{d}	0^{f}	–	0.0^{f}
WT exp YNB	20 ± 10^{cd}	2.2 ± 0.4^{d}	9.2 ± 5.6^{c}	13 ± 10^{ef}	2.3 ± 0.4^{de}	5.8 ± 4.9^{e}
WT stat YNB	16 ± 8^{d}	2.2 ± 0.4^{d}	6.9 ± 3.0^{c}	46 ± 23^{cd}	2.2 ± 0.4^{e}	20.0 ± 8.6^{d}
mnn9 exp YEPD	46 ± 20^{bc}	2.9 ± 0.4^{bc}	27.1 ± 14.2^{b}	44 ± 12^{cd}	2.8 ± 0.5^{cd}	22.6 ± 6.1^{d}
mnn9 stat YEPD	49 ± 14^{bc}	2.8 ± 0.4^{bc}	29.6 ± 11.3^{b}	54 ± 8^{bc}	3.1 ± 0.6^{bc}	33.9 ± 6.5^{c}
mnn9 exp YNB	91 ± 2^{a}	4.0 ± 0.6^{a}	74.5 ± 1.6^{a}	87 ± 5^{a}	4.0 ± 0.5^{a}	69.5 ± 5.8^{a}
mnn9 stat YNB	66 ± 13^{ab}	3.2 ± 0.7^{b}	42.8 ± 8.8^{b}	81 ± 9^{ab}	3.3 ± 0.6^{b}	54.3 ± 7.9^{b}

For each average, the respective standard deviation is added (mean ± S.D.). Each experiment was repeated twice: A and B. WT—wild-type yeast; exp—exponential growth phase; stat—stationary growth phase; YEPD—complete medium; YNB—minimal medium. Values in the same column (for each experiment) showing the same superscript letter are not significantly different ($p>0.05$).

stationary phase grown cells (see Tables 1 and 2—Experiment 1) with significant differences in four experiments (experiments A and B of WT-YEPD and mnn9-YNB). These differences occurred mainly due to the higher average individual length of nauplii (also significantly higher in four out of eight experiments) in these experiments.

However, in two of the experiments significantly higher *Artemia* total biomass was detected with stat-grown yeast, mainly due to the higher nauplii survival (three out of eight experiments revealed higher survival when nauplii were fed with stat-grown yeast).

3.1.3. Effect of media composition

In seven out of eight experiments, higher *Artemia* total biomass production was obtained with YNB-grown yeast, with significant differences found in five experiments (see Tables 1 and 2—Experiment 1). These results were due to higher nauplii survival (seven out of eight experiments) and average individual length (six out of eight experiments).

When the exp-grown WT yeast cells were fed to *Artemia*, total length values were not significantly different between both media. On the other hand, in all other cases (stat-grown WT, exp-grown and stat grown mnn9), total length values of *Artemia* were higher with YNB-grown yeast than with YEPD-grown yeast due to both higher survival and individual length (not always significant).

Summarizing, genetic background of yeast strains and, to less extent, growth medium are two parameters strongly determining the nutritional value of yeast for *Artemia*. The influence of the growth stage was not clear-cut. Yet, in both experiments (A and B), mnn9 cells grown to exponential phase in minimal medium proved to be the best feed as verified by the three measured characteristics (survival, individual length and total length).

Table 2
Experiment 1—number of experiments where differences (significant or not) of *Artemia* total biomass production (total length), survival and individual length occurred

Artemia	Higher or equal for WT	Higher for mnn9	Higher or equal for exp	Higher for stat	Higher or equal for YNB	Higher for YEPD
Total biomass production						
Significant	0	8	4	2	5	0
Not significant	0	0	1	1	2	1
Survival						
Significant	0	7	1	1	3	0
Not significant	0	1	4	2	4	1
Individual length						
Significant	0	7	4	0	4	0
Not significant	0	1	3	1	3	1

Comparisons were made between the use of the yeasts mnn9 strain and the wild-type (WT) strain as feed for nauplii; the use of yeast harvested in the exponential (exp) and stationary (stat) growth phase to feed *Artemia*; and the use of yeast cultured in YNB medium and YEPD medium as feed for the brine shrimp.

3.2. Characterization of live yeast as feed

The cells of the mutant mnn9 yeast seemed to have a higher AFDW content than the WT yeast cells (see Table 3) (although not significant when cells were grown in minimal medium and harvested in the exponential growth phase). This fact could have contributed to the considerable higher *Artemia* biomass production (total length). As mentioned above, the higher total length values (with mnn9 as feed) were in general the result of both higher survival and stronger individual growth. Compared to minimal medium, yeast cells grown in complete medium had generally an equal AFDW with the exception of exponential mnn9 cells that showed a significant higher AFDW when cultured in a complete medium. Anyhow, in general *Artemia* grew better on yeast cells cultured in minimal medium.

Finally, yeast cells harvested in the exponential phase showed only once a significant different AFDW with respect to stationary phase grown cells. In that case (mnn9 exp YNB), the yeast cells were considerably lighter (2.22 vs. 4.52 mg/10^9 cells in stationary phase), while *Artemia* growth in both experiments was significantly higher when fed exponential yeast cells, which was mainly due to a significant stronger individual growth, but also survival.

The comparison of the fatty acid (FA) composition of the two feeds that gave the most different *Artemia* results in this study (stat-grown WT cultured in YEPD and exp-grown mnn9 cultured in YNB) revealed almost no substantial differences. In both cases, mono-unsaturated (respectively 76.0% and 68.5% of total identified fatty acids) and saturated FA (respectively 21.6% and 29.3% of total identified fatty acids) mainly constituted their FA composition.

The cell wall composition of both yeasts was strongly dependent on experimental conditions (Table 3). Generally, compared to WT, mnn9 had a lower ratio of cell wall to cell mass. In terms of cell wall composition, mnn9 always showed significantly higher percentage of chitin and glucans and lower percentage of mannan than WT. Both yeast

Table 3
Average cell wall composition of the wild-type (WT) and mnn9 live strains of baker's yeast ($n=3$), cultured in a complete (YEPD) and minimal medium (YNB), harvested in the exponential (exp) and stationary (stat) growth phase and used as feed for *Artemia*

Yeast treatments	AFDW (mg/FT)	%Cell wall per cell mass	Percent of cell wall components			Ratio
			Chitin	Mannan	Glucans	Glucan/mannan
WT exp YEPD	0.19 ± 0.04^{b}	19.5 ± 1.2^{c}	1.2 ± 0.1^{a}	47.3 ± 1.7^{e}	51.5 ± 1.9^{a}	1.09 ± 0.08^{a}
WT stat YEPD	0.16 ± 0.01^{b}	23.6 ± 2.7^{cd}	1.0 ± 0.3^{a}	47.4 ± 1.9^{e}	51.4 ± 2.2^{a}	1.09 ± 0.09^{a}
WT exp YNB	0.23 ± 0.00^{b}	22.3 ± 2.7^{cd}	1.7 ± 0.2^{b}	43.4 ± 0.6^{d}	54.9 ± 0.5^{b}	1.27 ± 0.03^{b}
WT stat YNB	0.21 ± 0.00^{b}	23.3 ± 1.2^{d}	1.6 ± 0.1^{b}	42.1 ± 2.2^{d}	56.3 ± 2.3^{b}	1.34 ± 0.13^{b}
mnn9 exp YEPD	0.37 ± 0.09^{a}	13.1 ± 0.5^{a}	4.0 ± 0.7^{c}	23.1 ± 2.0^{c}	73.0 ± 2.4^{c}	3.19 ± 0.40^{c}
mnn9 stat YEPD	0.38 ± 0.02^{a}	16.9 ± 0.5^{b}	4.4 ± 0.4^{c}	22.9 ± 1.5^{c}	72.7 ± 1.9^{c}	3.19 ± 0.30^{c}
mnn9 exp YNB	0.19 ± 0.04^{b}	16.1 ± 1.3^{b}	8.7 ± 0.4^{e}	16.3 ± 2.1^{b}	75.0 ± 1.8^{c}	4.67 ± 0.77^{d}
mnn9 stat YNB	0.46 ± 0.07^{a}	23.2 ± 0.7^{d}	7.2 ± 1.0^{d}	13.2 ± 0.2^{a}	79.6 ± 1.1^{d}	6.06 ± 0.14^{e}

For each average, the respective standard deviation is added (mean ± S.D.). The average ash free dry weight (AFDW) of each feed added is expressed in mg/Falcon tube ($n=2$). Values in the same column showing the same superscript letter are not significantly different ($p>0.05$).

strains cultured in a minimal medium (YNB) always revealed significantly higher percentage of chitin and glucans and lower percentage of mannan when compared to yeast cultured in a complete medium (YEPD). Finally, the yeast growth stage had little effect on cell wall composition, with the exception of mnn9 cultured in YNB (exp-grown mnn9 had significantly higher percentage of chitin and mannan and lower percentage of glucans than stat-grown mnn9).

Significant correlations were found between the average total length of *Artemia* at the end of the experiments and the average percentage of each component present in the cell wall of yeast used to feed nauplii: chitin (Pearson test: experiment A, $r=0.975$, $p=0.000$, $n=8$; experiment B, $r=0.975$, $p=0.000$, $n=8$), mannan (Pearson test: experiment A, $r=-0.872$, $p=0.005$, $n=8$; experiment B, $r=-0.895$, $p=0.003$, $n=8$), and glucans (Pearson test: experiment A, $r=0.830$, $p=0.011$, $n=8$; experiment B, $r=0.859$, $p=0.006$, $n=8$) (Fig. 1).

3.3. Performance of Artemia fed dead and live yeast cells

The heat (autoclavation) and norvanol D treatments were sufficient to kill all yeast cells used to feed nauplii, as confirmed by the null growth of all yeast strains on YEPD agar plates. The applied γ-irradiation (10 kgy) was not sufficient to kill all yeast cells, yet, in all cases, more than 99.98% of cells were killed.

The results of the experiments comparing the use of either live or dead yeast as feed for *Artemia* are presented in Table 4 (Experiments 2, 3, 4 and 5). Irrespective of strain or growth phase used, autoclaved yeast cells always resulted in considerable and significantly higher *Artemia* survival rates compared to live cells. However, individual growth was lower or equal. In combination, total biomass production (total length) was usually higher (in six out of seven experiments, with significant differences in four out of eight experiments), except in one experiment with stationary mnn9 cells, where lower total length was registered for nauplii fed with autoclaved feed. These effects on *Artemia* were more pronounced with WT yeast cells as compared to mnn9 cells.

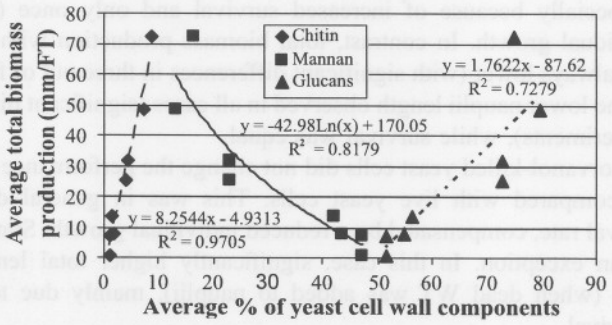

Fig. 1. Regression equation and respective r^2 values obtained between the average total biomass production (mm/FT) of both experiments (A and B) and the average percentage of each component of the yeast cell wall (chitin, mannan and glucans).

Table 4
Experiments 2, 3, 4 and 5—average survival (%), individual length (mm) and total length (mm per Falcon tube—FT) for *Artemia* nauplii together with the standard deviation (mean ± S.D.)

Yeast treatments	A			B		
	Survival (%)	Individual length (mm)	Total length (mm/FT)	Survival (%)	Individual length (mm)	Total length (mm/FT)
Experiment 2						
WT exp live	15 ± 4^{b}	2.3 ± 0.2^{a}	6.6 ± 1.2^{b}	18 ± 6^{c}	2.3 ± 0.5^{a}	7.8 ± 2.7^{b}
WT exp γ-irradiated 10 kgy	67 ± 9^{a}	2.0 ± 0.3^{a}	27.6 ± 5.1^{a}	58 ± 24^{b}	2.0 ± 0.3^{a}	23.4 ± 10.4^{a}
WT exp norvanol 20%	8 ± 4^{b}	2.1 ± 0.1^{ab}	3.7 ± 2.0^{b}	nd	nd	nd
WT exp autoclaved	87 ± 11^{a}	1.7 ± 0.2^{b}	30.9 ± 5.2^{a}	93 ± 5^{a}	1.7 ± 0.3^{b}	31.5 ± 1.5^{a}
Experiment 3						
WT stat live	3 ± 3^{c}	1.7 ± 0.1^{b}	0.9 ± 1.0^{d}	0^{c}	–	0.0^{c}
WT stat γ-irradiated 10 kgy	77 ± 5^{a}	3.1 ± 0.4^{a}	49.2 ± 3.1^{a}	36 ± 11^{b}	2.1 ± 0.3^{a}	15.4 ± 4.3^{b}
WT stat norvanol 20%	29 ± 5^{b}	2.1 ± 0.3^{ab}	12.4 ± 3.1^{b}	nd	nd	nd
WT stat autoclaved	74 ± 12^{a}	1.8 ± 0.3^{b}	27.6 ± 6.6^{c}	91 ± 11^{a}	1.8 ± 0.3^{a}	33.6 ± 5.2^{a}
Experiment 4						
mnn9 exp live	46 ± 20^{b}	2.9 ± 0.4^{a}	27.1 ± 14.2^{ab}	44 ± 12^{ab}	2.8 ± 0.5^{a}	22.6 ± 6.1^{a}
mnn9 exp γ-irradiated 10 kgy	43 ± 6^{b}	2.2 ± 0.4^{b}	22.2 ± 3.1^{b}	27 ± 9^{b}	2.4 ± 0.3^{b}	12.8 ± 3.4^{b}
mnn9 exp norvanol 20%	62 ± 6^{b}	2.3 ± 0.3^{b}	28.9 ± 2.2^{a}	59 ± 6^{a}	2.3 ± 0.3^{b}	26.9 ± 2.9^{a}
mnn9 exp autoclaved	94 ± 5^{a}	1.7 ± 0.2^{c}	32.5 ± 3.0^{a}	nd	nd	nd
Experiment 5						
mnn9 stat live	49 ± 14^{b}	2.8 ± 0.4^{a}	29.6 ± 11.3^{a}	54 ± 8^{b}	3.1 ± 0.6^{a}	33.9 ± 6.5^{a}
mnn9 stat γ-irradiated 10 kgy	34 ± 5^{b}	2.3 ± 0.4^{ab}	15.8 ± 0.8^{b}	45 ± 18^{b}	2.5 ± 0.4^{b}	22.0 ± 7.3^{b}
mnn9 stat norvanol 20%	48 ± 11^{b}	2.2 ± 0.3^{b}	21.9 ± 4.6^{ab}	63 ± 8^{b}	2.3 ± 0.4^{b}	30.5 ± 3.5^{a}
mnn9 stat autoclaved	91 ± 7^{a}	1.8 ± 0.3^{c}	35.2 ± 2.5^{a}	93 ± 6^{a}	1.7 ± 0.3^{c}	31.6 ± 1.3^{a}

Each experiment was repeated twice: A and B. WT—wild-type yeast; exp—exponential growth phase; stat—stationary growth phase; γ—gamma; nd—not done. Values in the same column (for each experiment) showing the same superscript letter are not significantly different. ($p>0.05$; $n=4$).

Artemia performed better when fed irradiated WT yeast cells in comparison with live yeast cells, especially because of increased survival and only once (WT-stat) due to increased individual growth. In contrast, total biomass production with irradiated mnn9 yeast cells was always lower (with significant differences in three out of four experiments) mainly due to the lower nauplii length observed in all cases (significant differences in three out of four experiments), while survival was equal.

In general, norvanol-killed yeast cells did not change the performance of *Artemia* (total length) when compared with live yeast cells. This was in general due to a slightly increased survival rate, compensated by a reduced individual growth. Stat-grown WT cells as feed were an exception. In this case, significantly higher total length values were measured once (when dead WT was added to nauplii), mainly due to a considerable increase in survival.

Artemia results obtained with irradiated mnn9 generally showed lower or equal total biomass production, mainly due to the low survival rates observed, when compared to nauplii fed with irradiated WT. A general improvement of nauplii performance was

observed when live and norvanol-killed mnn9 was added as feed, in comparison to the results of *Artemia* fed WT cells.

4. Discussion

In all experiments, independently of yeast culture conditions, mnn9-fed *Artemia* always performed better compared to the WT-fed nauplii (Tables 1 and 2). As a consequence of the mnn9 null mutations, the cell wall composition changes considerably (Table 3).

According to Klis et al. (2002), yeast cells have the ability to adjust the levels of the cell wall constituents, such as chitin, β-1,3 glucan and β-1,6 glucan, as an adaptation to the lack or reduced ability to produce mannoproteins, which is the case of the mnn9 yeast strain. Significantly decreased amounts of mannoproteins (from almost 40% to less than 10% of wet weight) and β-1,3 glucan (from 45–50% to 35% of wet weight) and increased amounts of β-1,6 glucan (from 10–15% to 30% of wet weight) and chitin (from less than 2% to 20% wet weight) are observed in the mnn9 mutant (Magnelli et al., 2002). Our results seem to confirm these changes, although the exact amounts were sometimes different, which is probably due to the method used to measure the chitin concentration in the cell wall. Indeed, the acid extraction used here tends to underestimate the amount of chitin between 10% and 30% in some experiments (J. François, unpublished data).

Feeding nauplii with yeast containing high levels of mannoproteins in the cell wall (WT strain) results in poor performance of *Artemia*. This correlation ($r=-0.85$ and $r=0.89$) seems to support the hypothesis of Coutteau et al. (1990), who stated that the nutritional value of live baker's yeast is not always consistent due to variation in the external mannoprotein layer making up the yeast cell wall. In fact, low mannase activity in the digestive tract of *Artemia* (reported by Coutteau et al., 1990) would prohibit digestion of yeast cells rich in cell wall-associated mannoproteins.

However, our study indicates that the amount of mannoproteins in the outer yeast cell wall is not the only factor associated with *Artemia* culture performance using yeast as feed.

The reduced production of mannoproteins is compensated by higher concentrations of β-glucans and chitin in the cell wall, resulting in a highly significant positive correlation between these two factors and *Artemia* total biomass production. Chitin is known to be very important to all crustaceans, including *Artemia*, since it is a major component of their exoskeleton (Horst, 1983). Therefore, a correlation between chitin concentration in the feed and nauplii performance seems logical.

Several factors are influencing yeast quality, putatively influencing digestibility for *Artemia*, such as age of cells (Shahin, 1972; Deutch and Parry, 1974), strain used (Killick, 1971), growth phase (Klis et al., 2002) and culture conditions such as the medium used, nature of the carbon source, nitrogen limitation, use of shake flasks or batch fermentors, pH, temperature and aeration/oxygen levels (Aguilar-Uscanga and François, 2003).

Independently of the medium used and the genetic background, *Artemia* survival, length and total length tended to be higher when nauplii were fed with yeast harvested in the exponential growth phase as compared to yeast harvested in the stationary growth phase. According to Klis et al. (2002), yeast cells entering stationary phase form thicker walls that are more resistant to β1,3-glucanase digestion and less permeable to macro-

molecules. The level of mannosyl phosphorylation of cell wall proteins increases at the late-exponential and stationary phase of cell growth (Odani et al., 1997). In addition, the number of disulfide bridges that link together the mannoproteins and glucan complexes in the wall also increases in the stationary phase (De Nobel et al., 2000). The phosphomannan complexes present in the outer yeast cell wall and the presence of disulphide bridges work as barriers affecting the penetration of the digestive/degradative enzymes to access the glucan complexes (present in the inner yeast cell wall) and the yeast cell content (Killick, 1971).

We confirmed in the present study that the cell wall mass of stationary phase cells is higher than in the exponential phase, although there was no significant difference in the polymer composition of the cell wall between the two growth phases of yeast. Hence, the reduced performance of *Artemia* with stationary phase yeast cells is more likely to be associated with the more intensive cross-linking between the major macromolecules of the yeast cell wall as they enter stationary phase, as reported by Aguilar-Uscanga and François (2003). Therefore, the nutritional value and digestibility of yeast by *Artemia* can possibly be significantly improved by manipulating the culture conditions of the yeast.

Both yeast strains cultured in YNB (minimal medium) and used as feed for *Artemia* always gave the best nauplii performance, when compared to yeasts cultured in YEPD (complete medium). According to Aguilar-Uscanga and François (2003), cell wall composition of yeast varies with the carbon source in the medium, and with the mode of cell cultivation. As reported by the same authors, glucan, mannan and chitin contents and glucan/mannan ratio of the yeast cell wall are lower in yeasts cultured in YNB than in YEPD. In the present study different results were obtained. The mannose content of YEPD-grown yeast was lower and the contents of chitin, glucans and the ratio glucan/mannan increased significantly. These differences were probably due to differences in the mode of cultivation, since in the first case, the cultivation was carried out in controlled batch bioreactors, while in the present study, the cultivation was done in shake flasks. Similar data as ours were found in an independent experiment (B. Aguilar-Uscanga and J. François, unpublished data). Anyhow, this subset of data on the effect of growth medium supports the general positive relation between glucan and chitin content in the yeast cell wall and the growth of *Artemia* on one hand and its negative relation with mannose on the other hand.

The performance of nauplii fed with exp-grown mnn9 cultured in YNB was clearly superior and similar to the results published by Coutteau et al. (1990), where brine shrimp were fed with xenic microalgae *Dunaliella tertiolecta*, which is considered one of the best feeds for *Artemia*. Hence, these results revealed that the mnn9 strain cultured in YNB to exp-phase is an excellent alternative for microalgae as feed for *Artemia*, since culturing yeast is less time- and labor-consuming than culturing algae. Apart from the high chitin and glucan and low mannose content, no obvious reason can be put forward to explain the results. The FAME analysis performed on exp-grown mnn9 cells cultured in YNB and on stat-grown WT cells cultured in YEPD (giving the worst result with *Artemia*) did not reveal any significant differences. Therefore, a difference in fatty acid profile is not likely to be the reason.

In previous studies on the effect of beneficial bacteria to *Artemia*, feed was sterilized either with antibiotics (Sick, 1976), heat treatment (Provasoli and Shiraishi, 1959;

Provasoli and D'Agostino, 1969; Douillet, 1987; Orozco-Medina et al., 2002) or γ-irradiation (Verschuere et al., 1999, 2000b). However, in all these studies, the effect of the sterilization method on the feed quality was not evaluated. The present study reveals that

Table 5
Effect of γ-irradiation, norvanol and heat treatment on different nutritional parameters of the feed (nl means that no information was found in literature)

Nutritional parameters	γ-Irradiation		Norvanol		Heat treatment	
	Effect	Reference	Effect	Reference	Effect	Reference
Molecule cleavage or change	Yes	Wu et al. (2002)	Yes	Zarate and Bradley (2003)	Yes	Korhonen et al. (1998)
Creation of free radicals	Yes	Wu et al. (2002)	No	nl	No	nl
Change in taste and colour	Yes	Pietranera et al. (2003)	Yes	nl	Yes	nl
Protein denaturation	Low	Ciesla et al. (2000)	Yes	Zarate and Bradley (2003)	Yes	Zarate and Bradley (2003)
Protein inactivation	Low	Pietranera et al. (2003)	Yes	Lerici and Manzocco (2000)	Yes	Prochaska et al. (2000)
Glycoprotein denaturation	No	Hallfeldt et al. (1995)	Yes	Hallfeldt et al. (1995)	Yes	Hallfeldt et al. (1995)
Vitamin destruction	Yes	ICGFI (1999)	Yes	Lieber (1989)	Yes	Prochaska et al. (2000)
Vital nutrients destruction	Low	ICGFI (1999)	Yes	nl	Yes	Prochaska et al. (2000)
Food energy content decrease	Yes	nl	Yes	nl	Yes	Prochaska et al. (2000)
Carbohydrate changes	Yes	Pietranera et al. (2003)	No	Bancroft and Gamble (2002)	Yes	Prochaska et al. (2000)
Lipid changes	No	Onyenekwe et al. (1997)	Yes	Bunout (1999)	Low	Prochaska et al. (2000)
Amino acid destruction	No	ICGFI (1999)	No	Gorham et al. (1982)	Yes	Prochaska et al. (2000)
DNA damage	Yes	Myung and Kolodner (2003)	Low	Bancroft and Gamble (2002)	Yes	Douillet (1987)
Water content decrease	Yes	Wang and Chao (2002)	Yes	Lerici and Manzocco (2000)	Yes	Masengi et al. (2001)
Mineral content changes	No	ICGFI (1999)	Low	Marsano and McClain (1989)	Yes	Watzke (1998)
Changes in fatty acid composition	Yes	Brito et al. (2002)	Yes	Bunout (1999)	Yes	Chantachum et al. (2000)
Changes or damage to cell wall	Yes	Kovács and Keresztes (2002)	–	nl	Yes	Neyens and Baeyens (2003)
Damage to cell membrane lipids and Increased permeability of the cell membrane phospholipid bilayer	Yes	Gaetke and Chow (2003)	Yes	Dickens and Callaway (information on website: http://www.k12.de.us/science/scivan/matthew.html)	Yes	Smelt et al. (2002)

the three sterilization methods tested have a negative or neutral effect on the feed quality (Table 4).

According to literature data gathered in Table 5, the three methods used in the present study to kill yeast cells have a strong potential to influence the nutritional value (by destroying some essential nutrients like vitamins, proteins and fatty acids) and to induce damages to cell membrane lipids (increasing the permeability of the membrane phospholipid bilayer). In addition, when feed is sterilized by autoclavation, more nutrients are destroyed than with a less destructive method such as γ-irradiation.

The high survival and low growth of *Artemia* fed with autoclaved yeast observed in this study was also recorded by Douillet (1987), Coutteau et al. (1990) and Orozco-Medina et al. (2002). According to Coutteau et al. (1990), the high survival could be due to improved digestibility after autoclavation. When yeast cells are heated, thermal denaturation/inactivation of proteins essential for cell wall rigidity may result in cell wall weakening, thus aggravating the effect of increased turgor pressure (Klis et al., 2002). The denaturation of mannoproteins present in the yeast cell wall also occurs when yeast is treated with norvanol (Lerici and Manzocco, 2000) and irradiation (Pietranera et al., 2003), improving digestibility of these yeast cells for brine shrimp in comparison to live yeast. This can explain the differences between nauplii performance fed with dead and live yeast cells, especially for the WT strain. The weak growth of *Artemia* fed autoclaved yeast (both strains), γ-irradiated yeast and norvanol-killed yeast (for the mnn9 strain, which is the most sensible strain to changes in osmotic pressure) could be due to reduced yeast biomass uptake. According to Coutteau et al. (1990), autoclavation causes lysis of part of the yeast cells, losing some of their intracellular nutrients (and affecting the water quality).

In studies where (new) probiotic bacterial strains are tested on *Artemia*, only the use of axenic live feed guarantees to avoid false positive results. The commonly used sterilization methods of the feed often yield *Artemia* of suboptimal quality due to the lack of essential nutrients in the dead feed added. Adding a bacterium to these *Artemia* can easily improve growth, but the bacteria may rather be a source of nutrients than an actual probiont. It is difficult to find a precise explanation for the results in which *Artemia* was fed with yeasts killed by the three sterilization methods, since several parameters change during the process, such as nutritional changes of yeast and variation of digestibility to nauplii (Provasoli and D'Agostino, 1962, 1969; Hernandorena, 1963, 1972a,b,c, 1974, 1976, 1979, 1990, 1991; Conklin and Provasoli, 1978).

5. Conclusion

Artemia performance (survival and growth) under axenic conditions can be perfectly controlled using yeast as feed. In addition, strongly divergent but reproducible culture results can be obtained by changing the yeast parameters, namely genetic background, growth phase and medium complexity. It is anticipated that the axenic *Artemia* growth test designed in this way will become an excellent tool to investigate the mode of action of bacteria in a standardized and conveniently simplified model of an aquatic food chain.

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